Dumbbell expression constructs for gene therapy

Description

The invention concerns a design principle for a minimalistic expression construct which contains no genetic information other than the information to be expressed , apart from promotor and terminator sequences which are necessary for the control of expression. Such minimal expression constructs are to be used for molecular-medical applications, specifically genetic vaccination, tumor therapy, and -prophylaxis.

The design principle is to be used for the construction of expression constructs for the expression of MHC-I or MHC-II presentable peptides, cytokines, or components of the regulation of the cell cycle, or for the synthesis of regulative RNA molecules and antisense RNA, ribozyme or mRNA-editing-RNA. Furthermore, an important aspect of the invention is that the construction principle allows for the covalent linking of the expression construct, e.g. with peptides, proteins, carbohydrates or glycopeptide ligands, as well as particles which allow for the transfer of the constructs into cells by ballistic transfer especially into dermis, muscle tissue, pancreas, and the liver.

The invention is to be used especially in two related fields: somatic gene therapy and genetic vaccination. These two meet in the field of immuno gene therapy of oncological conditions. Whereas classical gene therapy intends to substitute missing or defective genes, immuno gene therapy intends to activate the immune system of the patient against tumor specific antigens. In malignant melanoma and some other tumors, a number of tumorspecific antigens have been identified which can be recognized by cytotoxic T-

lymphocytes (Van den Eynde B. and Brichard V. G., Current Opinion in Immunology (1995) 7: 674-681). In most cases these are fragments of mutated proteins, which are either relevant for tumor development and –promotion, or are fragments of proteins from a changed metabolism of the tumor cell (Stüber et al., Eur. J. Immunol. (1994) 24: 765-768). In the case of melanoma, the presented peptides often derive from proteins from the melanocyte-specific differentiation. Approaches which make use of the activation of the immune system against such tumor specific antigens are in need of methods which enable the antigenic epitopes to be overexpressed in non-tumor cells, such as antigen-presenting cells (macrophages, dendritic cells). Alternatively, genes which control the expression of peptide-presenting proteins, such as CIITA or ICSBP are of great importance.

Laboratory experiments and clinical studies, in which such peptides have been used for the induction or amplification of a tumor specific cytotoxic response, concentrate on conventional vaccination protocols, in which the corresponding peptides are being used (Strominger J., Nature Medicine, (1995) 1:1.179-1.183). Alternatively, antigen-presenting cells such as dendritic cells, were incubated with high concentrations of such peptides. Thereby, the peptides originally present on the MHC-complex were exchanged for tumor specific peptides (Grabbe et al., Immunology Today (1995) 16:117-121).

The term genetic vaccination (immunization) describes the utilization of an experimental finding which first was debated as a scientific artefact, but has recently been corroborated in a number of biomedical problems (Piatak et al., Science 259 (1993): 1745-1749). If an expression plasmid for mammalian cells is injected into skin or muscle, there is, albeit in very low efficiency, an expression of the corresponding gene close to the injection site. If the expression product is a protein alien to the organism (xenogenic or allogenic protein), uptake and presentation of fragments of the expressed protein (oligopeptide) by antigen-presenting cells (APC) takes place, probably by way of local inflammation, Depending on local cytokine patterns and the type of cells

in which the plasmid is expressed (presentation by MHC-I or MHC-II), there is an induction of an immune reaction along the T_{H1} or T_{H2} pathway (Wang et al., Human Gene Therapy 6 (1995): 407-418), which eventually leads to the proliferation of cytotoxic T-cells or to the production of soluble antibodies. The transfection of dendritic cells with expression constructs for antigenic peptides ex-vivo is included in the term genetic vaccination in this context (Schadendorf et al. Molecular Medicine Today, 2 (1996): 144-145).

Such genetic vaccination avoids the numerous risks of conventional immunization approaches. Many approaches are known in gene therapy that are designed to effect therapeutic or prophylactic effects by the transfer of genetic information into cells. These approaches have not only been demonstrated in animal experiments, but also in numerous clinical studies in patients, an example being the so-called ballisto magnetic vector system (EP0686697 A2) for the transfection of conventional, plasmid-based expression constructs. The ballisto magnetic vector system was employed by the inventors of this application in three clinical phase I/II studies for the production of interleukin-7 (IL-7), interleukin-12 (IL-12) or granulocyte-macrophage-colony stimulating factor (GM-CSF) expressing tumor cells. In the case of expression of IL-12, separate expression constructs for the genes of the two IL-12 subunits were transferred ballisto-magnetically at the same time.

With the maturing of this new discipline, however, the methodological repertoire for gene therapy demands critical inspection. A fundamental aspect of this question is the sequence information contained in conventionally employed DNA constructs . If such expression constructs are to be employed in a great number of patients, and possibly more than once, safety aspects, especially those related to immunological concerns, will come to bear heavily. The conventionally used expression constructs are derivatives of eucaryotic expression plasmids. These have two fundamental disadvantages: their size, which inhibits fast transport into the cell's nucleus, and the presence of sequences which are not needed for the intended use. Expression

constructs used so far contain constitutively expressed genes, i.e. for resistance against cytostatica which serve as selection markers, and in some cases sequences for the episomal replication in the target cell. The expression of these genes leads to an unwanted background of transfected genetic information. Furthermore, apart from the promotor-gene-terminator structure which is to be expressed, these constructs carry at least the sequences needed for bacterial replication, since the plasmids are propagated in bacteria. These sequences are not needed for the intended use, either.

It is obvious that conventional expression constructs lead not only to the expression of the desired gene, but also to the biosynthesis of xenogenic proteins, even if their prokaryotic promotors show very low activity in mammalian cells. With longer or repeated application it can be assumed that the desired immune response is masked by such contaminating gene products, and significant immunological complications can occur.

Another problem in the application of gene therapeutic methods concerns the method by which the genetic material to be transferred is brought into the cell. For reasons of efficiency, immunological safety, and broad applicability across a wide spectrum of cell types, the method of ballistic transfer is preferred. A fundamental advantage of ballistic transfer, compared to alternative transfection methods, is that the method is applicable across a broad spectrum of different cells or tissues. Another disadvantage of methods currently used for the transfection of eukaryotic cells, such as electroporation or lipofaction, is that the treatment brings the substance to be transported only across the plasma membrane, the first barrier, which shields the cell from its environment. However, for most substances interacting with the regulative function of the cell, it is important to get from the cytoplasma across the nuclear membrane into the nucleus. This membrane is biophysically fundamentally different from the plasma membrane, and methods such as electroporation or lipofection do not lead to a passage through this membrane. The reason why these methods nonetheless lead to expression of recombinant nu-

cleic acid constructs transfected into the cells in a part of the cell population, is the fact that in the act of cell division, the nuclear membrane is rendered permeable. In consequence, methods such as electroporation or lipofaction only lead to transfection of cells which divide. Therefore these methods are not applicable to the transfection of many slowly or non-dividing cells, which can be interesting in the context of gene therapy, such as stem cells of the immune system or the heamatopoietic system, muscle cells, cells of exocrine or endocrine organs and their accompanying cells. The also commonly used and very efficient transfection method of retroviral transport of genetic material suffers the great disadvantage of targeting the tranfected cells for a possible cytotoxic reaction by the host organism, which probably limits the applicability of this method for gene therapeutic approaches.

The method of ballistic transfer has been used for the ex-vivo treatment of autologous and allogenic patient cells (Mahvi et.al.; Human Gene Therapy 7 (1996): 1535-43). However, when treating cells in tissue, a method which should be advantageous especially for the oncotherapeutic treatment of solid tumors or the mass prophylaxis against infections by genetic vaccination, the state of the art has disadvantages: The method of ballistic transfer makes use of DNA adsorbed to microprojectiles. When transfecting skin or other tissues, the penetration depth of the DNA constructs is lower than the penetration depth of the projectiles. DNA is desorbed soon after impact on the tissue. Only the uppermost tissue layer in the direction of the projectiles is transfected, although the projectiles themselves enter much deeper into the tissue. When transfecting solid tumor tissue (colon carcinoma, rectum carcinoma, reno-cell carcinoma and others), it has been found that, with suitable adaption of the parameters, the microprojectiles enter up to five cell layers deep into tissue slices. The transfected cells, however, (visible as fluorescent cells when transfected with a recombinant expression construct containing a green fluorescent protein from aequrea sectoria) were all found in the uppermost cell layer facing the impact of the microprojectiles. A more stable coupling of the DNA constructs to the surface of the microprojectiles would

be desirable in order to avoid the desorption of the substance to be transported. In this way only, the application of gene therapeutic approaches to solid tumors would be realistic, since only the transfection of tumor slices in the depth of the tissue enables a sufficient number of treated cells to be achieved. It is also imaginable that a combination of different coupling protocols enables the release of different genetic information within the same cell population at different timepoints. For these and numerous other applications, microparticles which bring the substance to be transported all the way into the hit cell and then make the substance available to the cell, would be very desirable.

US patent 5,584,807 (McCabe) describes an instrument in form of a gas pressure operated gun for the introduction of genetic material into biological tissue, in which gold particles are used as carrier material for the genetic information, without making reference to the nature of the genetic material in particular. US patents 5,580,859 and US 5,589,466 (Felgner) describe a method for the introduction of DNA into mammalian cells in the context of gene therapy. Naked DNA sequences coding for physiologically active proteins, peptides or polypeptides and are under the control of a promotor are injected directly into cells. Naked DNA refers to sequences that are free of other genetic material like viral sequences. DNA is expressed in these cells and serves as vaccine.

WO 96/26270 (Rhône-Poulenc Rorer S.A.) describes a circular double-stranded (supercoiled) DNA molecule, containing an expression cassette coding for a gene and controlled by a promoter and a terminator. This system is employed in vaccination in the context of gene therapy, also.

WO 96/32473 refers to a "dumb-bell oligonucleotide as sense inhibitors of the herpes simplex virus (HSV)". This discovery provides a description of closed, covalent, dumbell-shaped oligonucleotides, which contain a linkage point for ICP4-proteins of the herpes simplex virus

EP 0 686 697 A2 (Soft Gene) concerns a method for the enrichment of cells modified by ballistic transfer, and describes the technological background, the related problems, and the solutions found so far. The basic method of ballistic transfer is described herein. A device useful for the execution of this method is described in EP 0 732 395 A1.

The ballistic particles are gold particles with a diameter of either 1 μm or 1,5 μm (EP 0 686 697 A2), chosen depending upon the cell type. These gold particles are coated with superparamagnetic particles of roughly 30 nm diameter. The superparamagnetic particles at the same time furnish a useful surface for the coating with biomolecules. The use of magnetic particles enables subseqent separation.

Furthermore, dumbbell-shaped nucleic acid constructs are known that are characterized by the following features: They are short (10-50 bp double-stranded DNA) nucleic acid constructs, which were made for structure research or as double-stranded oligomers with improved nuclease resitence used for scavenging of sequence specific DNA ligands (Clusel et. al.; Nucleic Acids Res. 21 (1993): 3405-11; Lim et. al., Nucleic Acids Res. 25 (1997): 575-81).

Longer DNA molecules, which can exist throughout parts of their replication cycles as dumbbells, are known in nature as mitochondrial genomes of some species, such as ciliatae and yeasts (Dinuel et. al., Molecular and Cell Biology, 13 (1993): 2315-23). These molecules are about 50 kb in size and have a very complex genetical structure. Likewise, a closed covalent linear structure is known from vaccinia virus.

Peptide-nucleic acid-linkages with localization sequences are known for short DNA oligomers. Morris et al. . (Nucleic Acids Res. 25 (1997): 2730-36) describe the coupling of oligomers 18-36 base pairs in length, with a 27 amino acid residues containing peptide, which contains the nuclear localization se-

quence from SV40 as well as a signal peptide from HIV-gp41 responsible for the fusion with CD4-positive cells.

The use of peptide chains for crossing the endosomal membrane has been investigated by several groups. The 23 N-terminal amino acids of haemagglutinine were adsorbed by non-covalent interactions to expression plasmids in order to facilitate the uptake of these complexes into the cytosol after endosomal uptake (Plank et.al., J.Biol.Chem. 269, 12918, (1994)). The covalent attachment of antisense desoxyoligonucleotides to haemagglutinine peptide is described by Bongartz et al. (Nuc.Acids Res. 22, 4681, 1994).

Based on this state of the art, it is the objective of the invention presented here to develop an expression construct that contains only the information necessary to be expressed, and to provide means for the transport into a cell, which is to be treated therapeutically.

This objective is reached using the features of claims 1 and 13. According to the invention, double-stranded DNA-expression constructs, which are to be transported, are modified in such fashion that both anti-parallel strands of the DNA-polymer, containing the coding sequence and the promotor and terminator sequences necessary for its expression, are linked by loops of single stranded desoxyribonucleotides at both ends in such a way, that a continuous covalently closed molecule results. Preferably, said loop contains 3 to 7 nucleotides. In figure 1, such a construct is shown schematically. Such expression constructs are employed for the expression of MHC-I or MHC-II presentable peptides, cytokines, or components of the regulation of the cell cycle, or for the synthesis of regulative RNA-molecules, such as antisense-RNA, ribozymes or mRNA-editing RNA. Since the nucleic acid is covalently closed on both ends and no free hydroxyl-groups are available for nucleolytic cleavage, the molecule has a much higher stability against intra- and intercellular nucleases, and thus a longer halftime in the body or the cell. This advantage is especially important in the application in-vivo.

Furthermore, according to the invention, said loop linking the strands can contain one or more modified bases, said bases containing chemical functions, which allow the coupling of the molecule with a solid base, preferably amino-, carboxylic acid-, thiol-, or disulfide-modifications. Said modifications are covalently linked by known synthetic steps with corresponding carboxylic acid-, aldehyde-, amine-, thiole-, or other functions, or directly with a gold surface of a microprojectile for ballistic transfer. It can be imagined that a combination of different linking methods facilitates the release of a plurality of genetic information within the same cell population at different timepoints.

Apart from the aspect of easier chemical linking to the surface of the micro-projectile, said nucleic acid construct presents another advantage: nucleic acid constructs currently used in transfection in gene therapy, are produced in bacteria and carry, besides the sequences relevant in context of their therapeutical use, other sequences, which are only needed for the amplification of the nucleic acid constructs in bacteria. These sequences are an unknown risk for the patient who is to be treated, as it is not known whether and how these affect the organism. Such sequences, which not solely serve the primary objective of transfection in the target cells, can be excised from nucleic acids amplified in bacteria by restriction endonuclease digestion prior to transfection, and can be substituted by covalent linkage of short ends of desoxyribonucleic acid, said ends possibly being modified. According to another aspect of the invention, DNA to be transported into the cell can be obtained by polymerase chain reaction with chemically modified primers, so that the products of the polymerase chain reaction contain the chemical modifications needed for binding to the micro-projectile. An advantage of the construction principle according to the invention over current expression vectors is, that the resulting constructs contain only the sequence needed for the expression of the target gene.

Another aspect of the invention is that a loop of single stranded desoxyribonucleotides on either end of the molecules allows for the introduction of

chemical modifications in such a way that non-nucleic acid ligands can be covalently linked to the nucleic acid expression construct. This way for example, peptides needed for the nuclear localization of the expression constructs can be linked to the construct in such a way, that after entering the cytosol of a cell said construct is transported by the translocation apparatus of the cell into nuclear compartments where it can be transcribed,. Thereby, the constraints mentioned above concerning some transfection methods would be dealt with. Likewise the direct linking of the construct to peptides-, glycopeptide-, or carbohydrate-ligands which facilitate the entrance of the construct by cell-specific surface receptors is facilitated.

Specifically, according to the invention, double-stranded anti-parallel DNA expression constructs are modified in such a way that the ends of the double strands each contain a disulfid bridge linking the strands on both ends over covalently bonded alkyl groups bound to the 5'-end of one strand and the 3'-end of the other strand.

Any eucariotic promoter sequence can be employed for the control of transcription of the expression plasmid. Especially advantageous are short promoters with a high transcription rate transcribable in a multitude of cells, e.g. the "immediate early promotor" from cytomegalo virus (CMV) promoter . RNA-polymerase III-depending promoters such as the 7SK-promoter or the U6-promoter are of advantage for the transcription of genes coding for RNA. Such promoter sequences can result in the expression of short antisense-RNAs, ribozymes, and artificial mRNA in vivo. RNA-polymerase III produces significantly more copies of RNA than polymerase II and has an exact termination signal, a feature of special advantage. Said promotor sequences are characterized by their shortness, leading to small corresponding dumbbell expression constructs, a feature favourable to the entry of said expression constructs into the cell's nucleus.

The invention also concerns the transport of nucleic acids into cells. According to the invention, nucleic acids are transferred into cells by linking the nucleic acids to the surface of the micro-projectile carrying the nucleic acids into cells by adsorption, covalent or ionic interaction, in such a way that said nucleic acids are not or not completely desorbed upon passage of the micro-projectile through connected tissue into tissue fluid or cell layers, but remain linked to the microprojectiles until said microprojectiles come to rest with said nucleic acids in the target cells. This has the advantage that the substance is provided to a cell either in its bound state, or is desorbed in a process slow in comparison to the time of entry and is provided to the cell in a desorbed state, depending on the mode of binding.

The binding of thioles or disulfides to gold surfaces is well researched and described (G. M. Whitesides et al., Langmuir 10 (1994): 1825-1831). The nucleic acids to be transported are adsorbed preferably to micro-projectiles made of gold, however, by providing the nucleic acids with thiole or disulfide groups by covalent modification with publicly available reagents, and then adsorbed through their thiole or disulfide groups, or covalently bound to the micro-projectile by anodic oxidation of the thiole or disulfide functions employing the gold of the micro-projectile as anode. According to the invention, the substance to be transported is bound by sulfur-gold linkage or by disulfide linkage to the micro-projectile. When employing micro-projectiles made of gold, the substance to be transported is modified by molecules containing thiole groups or disulfide bridges, if it does not already contain thiole or disulfide groups able to bind by themself. Said substance is then bound to the gold surface of the micro-projectile by chemisorption. The resulting gold-sulfur linkage is sufficiently strong to carry the molecules to be transported through several cell layers.

Since the cell contains molecules comprising thiole groups, above all the ubiquitous glutathion, an equilibrium reaction of the thiole groups on the gold surface leads to the slow desorption of the chemisorbed molecule from the

surface of the micro-projectile. Thereby, the transported substance is freely available to the cell after desorption. Furthermore, according to the invention, when employing micro-projectiles made of gold, the molecule to be transported containing thiole or disulfide bridges can be bound covalently to the micro-projectile by anodic oxidation of the thiole or disulfide functions employing the gold micro-projectile as anode.

According to the invention, when employing micro-projectiles made of oxidic ceramics, glass ceramic, or glass, the molecule to be transported is bound by ester, amide, aldimine, ketal, acetal or ether linkage, or other functionalities known to the organic chemist for binding of molecules to a solid surface. The numerous silane reagents used for the modification of silicon oxyde phases can be employed here.

The invention is used for ex-vivo gene therapy. Preferably, interleukin-7 (IL-7) and interleukin-12 (IL-12) proteins and their subunits are expressed, as are interleukines, granulocyte-makrophage-colony-stimulating factor (GM-CSF), cell surface antigens and ligands of immune controlling or –modifying lymphocyte antigens like CD40, B7-1, and B7-2, proteins of the MHC-complexes I or II or ß-2 microglobulin, interferone consensus sequence binding protein ICSBP, CIITA, Flt3, or entire proteins or fragments thereof of presentable epitopes from tumor specific expressed mutated or non-mutated proteins, e.g. Ki-RAS-fragments, p16 and p53, or bcr-abl product. The use of micro-projectiles which are all linked with constructs of the same type is preferred, but cocktails (mixtures) of micro-projectiles which are each linked to different constructs are possible, as are micro-projectiles which are each linked with a cocktail of different constructs.

The desoxyribonucleic acid construct according to the invention is preferably employed as vaccine for the treatment of infectious diseases in humans and animals, e.g. malaria and influenza.

More advantageous features are contained in the subclaims. The invention is depicted in the attached figures and is described more closely in the following examples.

Figure 1 shows a schematic outline of the construction concept, whereas Fig. 1.1. shows the confirmation of the covalently closed phosphate-sugar-backbone; the number of base pairs depicted does not necessarily show the length of the constructs but only serves as example of the principle; Fig. 1.2. shows the functional structure of one of several possible expression constructs;

Figure 2 shows the synthesis of the constructs schematically.

Example 1 [Synthesis of the expression constructs]:

The fundamental construction principle is depicted schematically in Fig. 2 and is as follows:

1.1 Synthesis from vector:

The gene to be expressed, e.g. granulocyte-macrophage stimulating factor (GM-CSF), is amplified from cDNA using suitable primers by PCR (Fig. 2 (1)) and recombined into a suitable plasmid vector (Fig. 2 (2)). After sequencing and confirmation of the target sequence, the sequence to be expressed is amplified from said plasmid vector by means of two primer sequences (oligodesoxynucleotides carrying on the 5'-end of their sequence restriction enzyme recognition sites) (Fig. 2 (3)). The resulting amplification product is digested with said endonucleases, for which a recognition site was provided on said primers. After isolation of the amplified fragment from an agarose-gel,

said fragment is recombined into an expression plasmid, which is amplifiable in bacteria, and in which the gene to be expressed is located in the desired orientation in the context of the sequences controlling expression contained in said expression plasmid (Fig 2 (4)).

Said expression plasmid is amplified in bacteria and isolated according to methods known in the art. After digestion with restriction endonucleases, the recognition sites of which are flanking the sequence which is to be contained in the dumbbell-shaped expression constructs, the restriction fragments are separately isolated by methods of anion exchange chromatography (Fig. 2 (5)), and are subsequently ligated to hairpin-forming self-hybridizing oligodesoxynucleotides (short DNA-molecules obtained by automated chemical DNA synthesis, which can form stem-loop-structures based on their self-complimentarity; these molecules will later form the covalently closed ends of the dumbbell-shaped DNA-molecules), which contain an single-stranded overlap compatible with the overlap of the construct obtained by the digestion with endonucleases (Fig. 2 (6)). After separation of excess hairpin desoxynucleotides by anion exchange chromatography, the constructs according to the invention are obtained.

1.2. Synthesis from PCR-product

Alternatively, the construct is amplified directly by polymerase chain reaction, the primer oligodesoxynucleotides carrying recognition sites for restriction endonucleases on the 5-prime ends of their sequence (Fig. 2 (7)). After separation of the primers by anion exchange chromatography, the resulting amplificate is digested with said endonucleases, for which a recognition sequence was provided on said primer oligodesoxynucleotides. After separation of the smaller restriction fragments, the construct is ligated to short hairpin-formed self-hybridizing oligodesoxynucleotides, said hairpin-formed desoxynucleotides providing a overhang able to hybridize to the overhang resulting from the restriction enzyme digestion of the construct (Fig. 2 (8)). After separation

of excess hairpin-desoxyoligonucleotides by anion exchange chromatography, the constructs according to the invention are obtained.

An expression construct consisting of the sequence for gm-csf under control of the "early immediate promotor" from CMV and the polyadenylation sequence from SV40, was obtained from plasmid mtv-GM-CSF by complete digestion with EcoRI and HindIII. The smaller fragment (1290 bp) was isolated by anion exchange chromatography (stationary phase Merck fractogel EMD-DMAE; 25 mM Tris/HCl pH8; 0-1 M NaCl), and after concentration and desalting, was ligated with a 200-fold molar excess of 5´ phosphorylated hairpin-desoxyribonucleotides AATTCGGCCGGCCGTTTTCGGCCGGCCG and AGCTTGGCCGGCCGTTTTCGGCCGGCCA (TIB Molbiol, Berlin) in the presence of 25 U/ml T4-DNA-ligase overnight at room temperature. The reaction was stopped by heating to 60°C. The construct ligated to the desoxyoligoribonucleotide was separated from excess desoxyoligoribonucleotide by anion exchange chromatography, concentrated by ethanol precipitation, dissolved in water and applied to sterile primary colon carcinoma cells using the ballistic transfer according to a published method.

Example 2 [*in vivo* expression]:

Ballistic transfer of GM-CSF into K562:

30 µl of a suspension of gold particles (1.6 µm diameter, supplied by Bio-Rad, Hercules, CA, USA, concentration of the suspension: 30 mg/ml) are transferred to a macro carrier-polymer sheet (Bio-Rad). The gold is allowed to sediment, and the supernatant is cautiously removed. Onto the wetted surface, 30 µl of a 1+3 mixture of a suspension of colloidal magnetic particles (mean diameter 65 nm; Miltenyi GmbH, Bergisch-Gladbach, Germany; used as supplied; concentration unknown) and GM-CSF-expression-dumbbell-construct (example 1) are pipetted. The sedimented gold is re-suspended in said mixture and allowed to re-sediment. The supernatant liquid is removed

and the gold particles are allowed to dry. 300 µl polylysine are transferred to the center of a petri-dish (3,5 cm), allowed to rest for 30 min and washed off with PBS-medium. 100.000-200.000 cells (erytroleukemia cell line K562) are transferred onto the polylysine-coated surface of the petri-dish in 300 µl RPMI-medium (10% FCS), and allowed to rest for 10 min. 2 ml RPMI-medium (10% FCS) are added, and the cells are incubated 1-2 h in an incubator.

Ballistic transfer is conducted according to the manufacturers with a Biolystic PDS 1000/C (Bio-Rad, Hercules, CA, USA). The rupture disk employed corresponds to a pressure of 1100 psi. The pressure of the vacuum cell is 508 mm Hg. Magnetic separation is conducted as published (EP 0732 395A1); control of successful transfection is performed by GM-CSF-ELISA.

Example 3 [Example for synthesis of a expression-construct with nuclear localization sequence]:

An expression construct consisting of the gene for a green fluorescent protein under control of the early immediate promoter from CMV and the polyadenylation sequence of SV40 (pEGFP, Clontech Inc.) was obtained by restricition enzyme digestion with EcoRI and HindIII. The smaller fragment was isolated by anion exchange chromatography (stat.phase: Merck Fractogel EMD-DMAE; 25 mM Tris/HCl pH 8; 0-1M NaCl) and ligated after concentration and desalting with a 200-fold molar excess of 5´-phosphorylated hairpin-desoxyoligoribonucleotides AATTCGGCCGGCCGTXTCGGCCGGCCG and AGCTTGGCCGGCCGTXTCGGCCGGCCA in the presence of 25 u/ml T4-DNA-Ligase overnight at room temperature (X signifies the peptide modification: Amino-Uracil coupled to the peptide by amide function (TIB-Molbiol, Berlin)) The reaction was stopped by heating to 60°C. The construct ligated to the amino-desoxy-uracil-modified desoxyoligonucleotide was separated from excess hairpin desoxynucleotides by anion exchange chromatography, concentrated by ethanol precipitation and dissolved in water. 1 µg of the

thiol-modified construct was incubated with 1 mg micro-projectiles (spherical gold, mean diameter 1 μm, Bio-Rad, Hercules, CA) in water over night at room temperature. The gold particles were washed twice with water and applied to adherent ceratinocytes with the ballistic transfer according to the known procedure.

Example 4 [Synthesis of nucleic-acid-modified gold particles]:

An expression construct, which consists of the sequence for gm-csf under control of the "early immediate promoter" from CMV and the polyadenylation sequence from SV40 was excised from the plasmid mtv-gmcsf by complete digest with EcoRI and HindIII. The smaller fragment (1290 bp) was isolated by anion exchange chromatography (stat. phase : Merck Fractogel EMD-DMAE; 25 mM Tris/HCl pH 8; 0-1 M NaCl) and following concentration and desalting ligated to a 200-fold molar excess of 5´phosphorylated hairpin desoxyoligoribonucleotides AATTCGGCCGGCCGTXTCGGCCGGCCG and AGCTTGGCCGGCCGTXTCGGCCGGCCA (X specifies the thiol modifier C6 S-S (TIB-Molbiol, Berlin)) in the presence of 25 u/ml T4 DNA Ligase and incubated over night at room temperature. The reaction was stopped by heating to 60 °C. The construct ligated to the thiol desoxyribonucleotide was separated from excess thiol modified desoxyribonucleotide by anion exchange chromatography, and resolved in water. 1 μg of the thiol modified construt was incubated over night with 1 mg microprojectiles (shperical gold, mean diameter 1 μm, Bio-Rad, Hercules, CA) in water. The gold particles were washed twice with water and used for ballistic transfer into sterile primary coloncarcinoma cells (see Expl. 5).

Example 5 [Ballistic transfer to solid tumor tissue]:

Sterile primary colon carcinoma tissue was removed surgically and cooled on ice. Necrotic parts and connective tissue is removed as much as possible. Pieces of ca. 1 cm^2 surface are excised from the tumor, washed in ice-cold

PBS and fixated on the sample holder of a tissue slicer (vibratome 1000 sectioning system; TPI, St. Louis, Missouri) with tissue glue. The tumor is sliced into slices of 500 µm thickness. The slices are stored in ice-cold PBS and transfected as soon as possible. 30 µl of a suspension of GM-CSF-expression construct-coated gold particles and colloidal magnetic particles (mean diameter: 65 nm – Miltenyi GmbH, Bergisch-Gladbach) are pipeted onto a macro carrier polymer sheet (Bio-Rad). The gold is allowed to sediment, the supernatant removed and the gold particles are allowed to dry. The procedure of ballistic transfer is identical with the procedure described in example 2. Both sides of the tumor slice are transfected. After transfection, the slice is passed twice through a cell sieve, and the cells are separated as described.

Magnetic separation is performed according to the published protocol (EP 0 732 395 A1); the success of the transfection is controlled by GM-CSF-ELISA.

Example 6 [Synthesis of nucleic acid-modified aluminum particles]:

5 µg of an expression construct consisting of the gene for a green fluorescent protein under control of the early immediate promoter from CMV and the polyadenylation sequence of SV40 (pEGFP, Clontech Inc.) was obtained by restricition enzyme digestion with EcoRI and HindIII. The smaller fragment was isolated by anion exchange chromatography (stat.phase: Merck Fractogel EMD-DMAE; 25 mM Tris/HCl pH 8; 0-1M NaCl) and ligated after concentration and desalting with a 200-fold molar excess of 5´-phosphorylated hairpin-desoxyoligoribonucleotides AATTCGGCCGGCCGTYTCGGCCGGCCG and AGCTTGGCCGGCCGTYTCGGCCGGCCA (Y signifies the carboxylic acid modified thymidinedesoxynucleotide (TIB-Molbiol, Berlin)) in the presence of 25 U/ml T4-DNA-Ligase overnight at room temperature

1g aluminiumoxyde particles (mean diameter 1,0 µm) were refluxed in a solution of tri-etoxaminopropylsilane in toluene (2%) overnight. The solid matter is

filtrated, washed with toluene and ethanol, dried and ground. 5 mg of the resulting amino-modified aluminiumoxyde are reacted in 100 ml aqueous carbonate buffer (pH 8,0) with 4 μg of the carbonic-acid-modified construct in the presence of 50 μM 1-ethyl-3-(3-dimethylaminopropyld)carbodiimide and 50 mM N-hydroxysuccinimide for 2 h at room temperature. The resulting nucleic-acid modified microparticles can be transported to cells by acceleration in a suitable apparatus, as described in DE 195 10 696 and EP 0 732 395 A1, whereby the information contained in the transported constructs is made available to the cells.

Claims

1. Deoxyribonucleic acid construct for transcription of RNA-molecules, characterized by a circular strand of deoxyribonucleic acid comprising a partly complementary, antiparallel base sequence, so that a dumbbell-shaped construct is formed,

   - in which the complementary, antiparallel base sequence in the essential comprises a promotor sequence, a coding sequence and a polyadenylation signal or another RNA-stabilizing signal,

   - and the non-complementary sequence comprises two loops of single-stranded deoxyribonucleic acid, which covalently join the 5´- and 3´-ends of the complementary, antiparallel strands.

2. Deoxyribonucleic acid construct according to claim 1, characterized by that said loops consisting of three to seven nucleotides, and in which one or several of said nucleotides are covalently modified by carboxylic acid-, amine-, thiole- or aldehyde functionalities.

3. Deoxyribonucleic acid construct according to claim 2, characterized by that said chemically modified nucleotides is being linked to a peptide leading to the directed transport of the construct into the nucleus.

4. Deoxyribonucleic acid construct according to claim 2, characterized by that said chemically modified nucleotides is being linked to a peptide enabling liberation of the construct from the endosome.

5. Deoxyribonucleic acid construct according to claim 1, characterized by using a 7SK promoter as said promoter.

6. Deoxyribonucleic acid construct according to claim 1, characterized by using a CMV promoter as said promoter.

7. Deoxyribonucleic acid construct according to at least one of the claims a to 6, coding for interleukine-7.

8. Deoxyribonucleic acid construct according to at least one of the claims 1 to 6, coding for interleukine-12 or one or several of its constituting sub-units.

9. Deoxyribonucleic acid construct according to at least one of the claims 1 to 6, coding for gm-csf.

10. Deoxyribonucleic acid construct according to at least one of the claims 1 to 6, coding for p16 or p53 protein or fragments thereof.

11. Deoxyribonucleic acid construct according to at least one of the claims 1 to 6, coding for peptide fragments of mutated ki-ras, mutated p53 or bcr-abl translocation product with a length of between 10 and 100 amino acids.

12. Micro projectile for ballistic transfer of deoxyribonucleic acid constructs into cells according to one or several of claims 1 to 11, in which the substance to be transported is linked by adsorption or covalent or ionic binding in such a way to said micro projectile that the substance to be transported upon passage of the micro-particle through connective tissue, the extra cellular liquid or cell layers is not or not completely desorbed, but remains bound to said micro projectile until the substance to be transported rests along with said micro projectile in the target cell.

13. Micro projectile according to claim 12, characterized by that its material being gold, micro crystalline gold, oxide ceramic, glass ceramic or glass and said nucleic acid to be transported is bound covalently by thiole- or disulfide moieties, ester-, amide-, aldimine-, ketale- or acetale- or ether functionalities to said micro projectile.

14. Micro projectile according to claim 12 or 13, characterized by that said micro projectile being made out of an electrically conducive material and said nucleic acid being linked to said micro projectile by electrochemically coupling of disulfide or thiole moieties, employing the micro projectile as electrode.

15. Micro projectile according to claim 12 to 14, characterized by that said micro projectile being of the size of 0,3 μm to 3 μm.

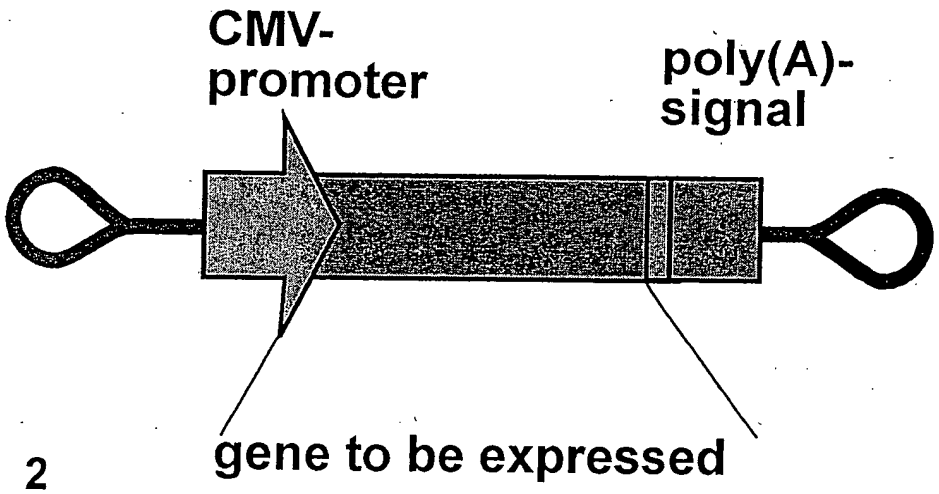
16. Use of a nucleic acid construct according to one or more of the claims 1 to 11 in *ex-vivo* gene therapy.

17. Use of a micro projectile according to one or more of the claims 12 to 15 in *ex-vivo* gene therapy.

18. Use of said deoxyribonucleic acid constructs according to one or several of the claims 1 to 6 for producing a vaccine for prevention of infectious disease in humans or animals.

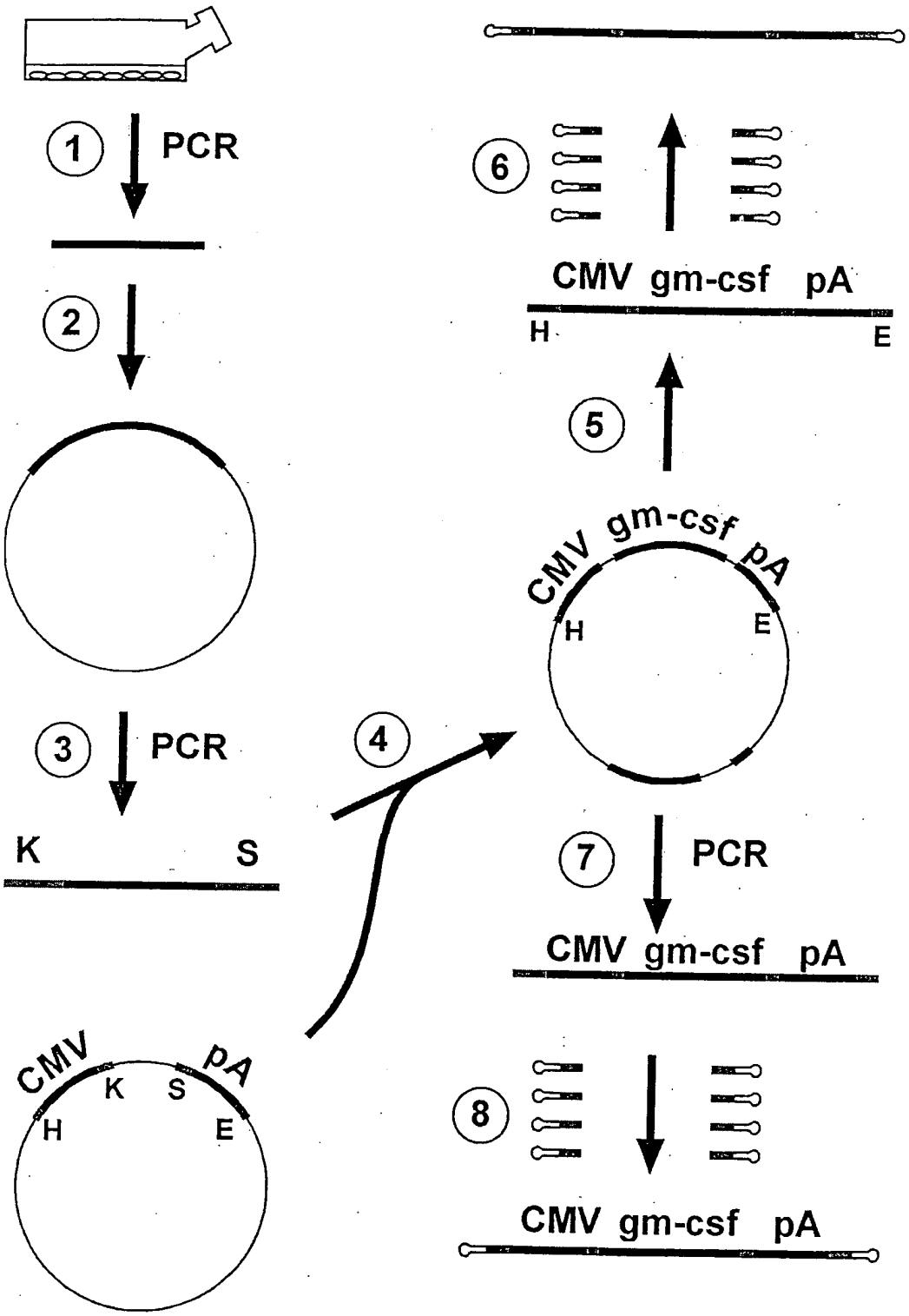
Fig. 1

1



2

Fig. 2


1
PCR
2
3
PCR
K
S
4
CMV
pA
K
S
H
E
CMV gm-csf pA
H
E
5
6
CMV gm-csf pA
H
E
7
PCR
CMV gm-csf pA
8
CMV gm-csf pA